Supporting Information

Analysis of enantioselective biotransformations using a few hundred cells on an integrated microfluidic chip

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1. Chemicals and reagents

Yeast extract, peptone, sodium chloride, ampicillin sodium salt, kanamycin sulfate, trypan blue, methanol (HPLC grade), ethanol (96%), acetonitrile (LC-MS grade) and sodium hydroxide were purchased from Carl Roth (Karlsruhe, Germany). Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was obtained from Bioline (Luckenwalde, Germany). Phosphate buffered saline (PBS), boric acid, 1,2-epoxy-3-phenoxypropane, (*S*)-2-oxiranylanisole, 2-[(3-methylphenoxy)methyl]oxirane, 4-chlorophenyl-glycidyl-ether and lysozyme (from chicken egg white) were purchased from Sigma-Aldrich (Steinheim, Germany). Heptakis(2,3-dihydroxy-6-sulfato)-β-cyclodextrin heptasodium salt (H6SBCD) was acquired from TM Chemicals (Deer Park, TX, US), 3-phenoxy-1,2-propanediol from TCI Europe (Zwijndrecht, Belgium), (*S*)-3-phenoxypropane-1,2-diol from Maybridge (Loughborough UK), glycidyl-4-methoxyphenyl-ether from Alfa Aesar (Karlsruhe, Germany), [(4-fluorophenoxy)methyl]oxirane from Acros Organics (Geel, Belgium) and disodium tetraborate decahydrate from Merck (Darmstadt, Germany). The gels for SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis, 4-15% Mini-Protean TGX precast gels, 4561083) were bought from BioRad. The bovine serum albumin (BSA) standard and the PageBlue™ Protein Staining Solution (24620) were obtained from Thermo Fisher Scientific. Ultrapure water was generated by a TKA Smart2Pure purification system (Niederelbert, Germany). All chemicals were purchased at highest available purity (> 96%) and used without further purification. All solutions except the samples were filtered with 0.2 µm pore size nylon syringe filters (Carl Roth, Karlsruhe, Germany) before their introduction into the chip system. Stock solutions of epoxides and diols were prepared freshly in water, acetonitrile or a mixture of both, which were then further diluted in borate buffer or PBS buffer.

2. Microbiology and molecular biology

E. coli BL21-Gold(DE3) (pQE60, ampicillin resistance; pREP4, kanamycin resistance) expressing ANEH-WT or the enzyme mutant ANEH-LW202 (L215F/A217N/R219S/L249Y/T317W/T318V/M329P/L330Y/C350V) [1], were cultivated following standard procedures (control samples included *E. coli* BL21-Gold(DE3) with a plasmid but without the ANEH gene). Briefly, antibiotics were added to the cultivation medium (LB medium: 5 g/L yeast extract, 10 g/L peptone, 10 g/L NaCl) with a final concentration of 100 µg/mL ampicillin and 50 µg/mL kanamycin. The cells were cultivated at 37 °C for 10 h to obtain pre-cultures. A pre-culture was then used to generate a main culture by transferring a small volume of the culture (1/1000). After approximately 3 h, the optical density value at a wavelength of 600 nm (OD_{600}) of the main culture reached 0.6 to 0.8, as determined by an Ultrospec 10 Cell Density Meter (GE Healthcare). This cell concentration is the optimal to induce ANEH expression using IPTG at a final concentration of 1 mM and at a temperature of 30 °C. Before using the IPTG-induced main-culture for further experiments, the OD_{600}-value was checked for a diluted culture (50 µL cell culture and 950 µL LB medium). For the determination of the cell concentration 50 µL of an exemplary cell culture of *E. coli* BL21-Gold(DE3) ANEH-WT and 200 µL PBS buffer were added to 750 µL 5.2 mM trypan blue solution with 154 mM NaCl. The cells were incubated for 3 min at 30 °C and inserted into a *Neubauer improved* counting chamber. After a sedimentation time of 1.5 min photos were taken and cells could be counted. After each filling procedure the chamber was cleaned carefully with ethanol. The double determination was performed twice. $5.7 \cdot 10^6$ cells/µL with a standard deviation of $0.9 \cdot 10^6$ cells/µL were calculated aligned to an OD_{600}-value

(1) Reetz, M. T.; Wang, L. W.; Bocola, M.; *Angew. Chem., Int. Ed.* **2006**, *45*, 1236-1241.

for a diluted cell suspension of 0.30 ± 0.02 (50 µL cell culture and 950 µL LB medium). Further a PBS buffer washed cell suspension was determined twice as well. All cell washing procedures used 1 mL of a cell sample centrifuged at 4000 rpm for 5 min. The supernatant was removed and 1 mL PBS buffer was added. After another identical centrifugation step the cells were resuspended in fresh PBS buffer solution. The amount of cells remaining after a washing procedure was calculated to be 87% ± 9%.

3. Microchip electrophoresis

Microchip electrophoresis (MCE) was performed using a fused silica microfluidic chip with an integrated microreactor structure manufactured by iX-factory (Dortmund, Germany) according to the layout developed by our group (Figure S1).

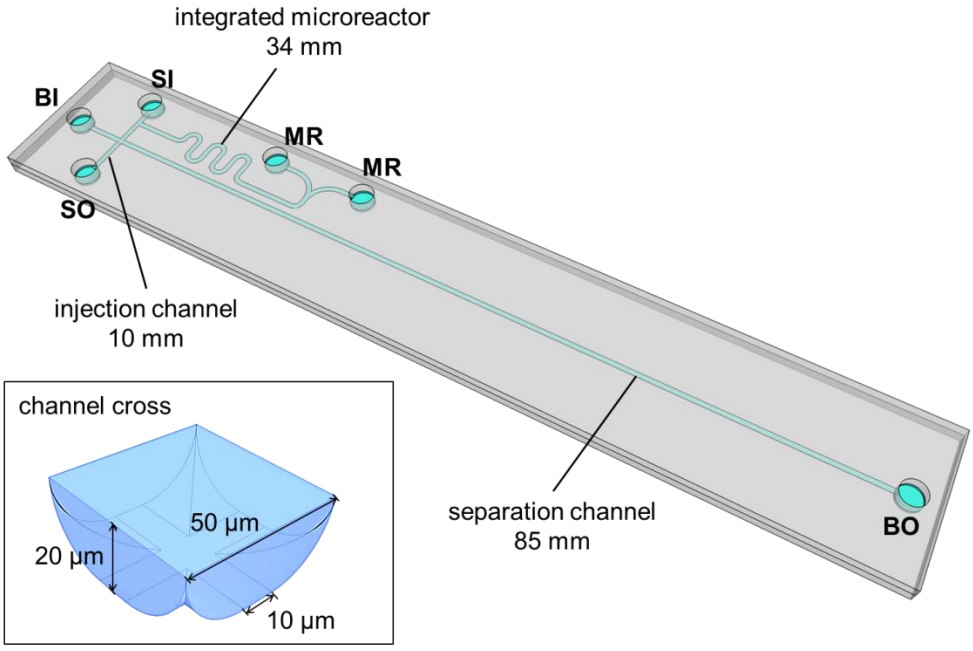

Figure S1. Schematic drawing of the microfluidic chip with integrated functionalities and channel dimensions as well as detailed injection and separation channel cross. BI, buffer inlet vial; BO, buffer outlet vial; MR, microreactor vial; SI, sample inlet vial; SO, sample outlet vial.

The chip layout included a 10 mm long injection channel crossing an 85 mm long separation channel with access cavities at each end of the channels. All tub-shaped microchannels were defined by photolithography and wet chemical etching to 20 µm in depth and 50 µm in width. The detailed dimensions were determined via microscopic images from top and in cross section leading to an upper edge channel size of 49.4 µm ± 1.7 µm, a lower edge size of 12.7 µm ± 0.5 µm and a channel depth of 19.3 µm ± 0.7 µm (n = 4). For chip electrophoresis the cavities designated sample outlet (SO), buffer inlet (BI) and buffer outlet (BO) as well as all microchannels were filled with separation buffer. Then, the sample was placed in the sample inlet cavity (SI) and a voltage-controlled pinched injection program[2] was performed. An electrical field was provided by a bipolar four-channel high-voltage power supply (HCV 40M-10000, FuG Elektronik, Rosenheim, Germany) connected to the microchip via a homemade PMMA plate with integrated platinum/iridium electrodes. On-chip separations were performed in borate buffer (125 mM, pH 8.5) at an effective field strength of 400 V/cm and a separation length of 7.4 cm. For chiral separations 15 mM H6SBCD was added to the buffer system operating as chiral selector. Peak assignments were verified by standard addition of pure substances ((*S*)-2-oxiranylanisole, (*S*)-3-phenoxypropane-1,2-diol to racemic substrate 1,2-epoxy-3-phenoxypropane **1** and product 3-phenoxy-1,2-propanediol **2**) (Figure S2).

(2) Jacobson, S. C.; Hergenroder, R.; Koutny, L. B.; Warmack, R. J.; Ramsey, J. M. *Anal. Chem.* **1994**, *66*, 1107-1113.

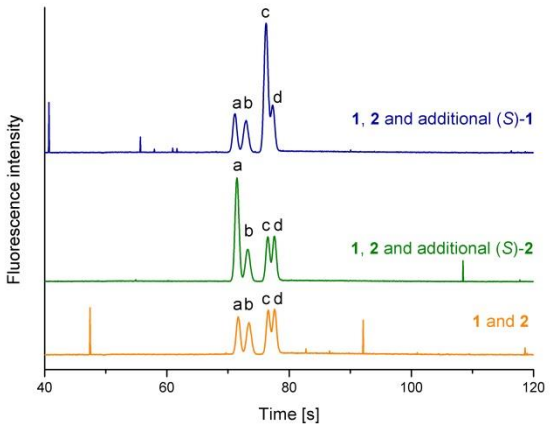

Figure S2. Electropherograms for peak assignments by standard addition of enantiomers. Substrate **1** and product **2** (yellow), **1** and **2** with additional (*S*)-**2** (green) and **1** and **2** with additional (*S*)-**1** (blue). Signal a: (*S*)-**2**; signal b: (*R*)-**2**; signal c: (*S*)-**1**; signal d: (*R*)-**1**.

Between measurements, the chip was thoroughly cleaned by rinsing it with 0.1 M NaOH, H_2O and separation buffer (125 mM borate buffer, pH 8.5). If the inserted sample contained biological material, MeOH was used first to clean all cavities and channels.

4. Time-resolved fluorescence detection and analysis

In contrast to our previous work[3], herein we used a significantly advanced detection setup based on confocal microscopy and deep UV fluorescence excitation for laser induced fluorescence (LIF) detection (Figure S3).

(3) Belder, D.; Ludwig, M.; Wang, L. W.; Reetz, M. T. *Angew. Chem., Int. Ed.* **2006**, *45*, 2463-2466.

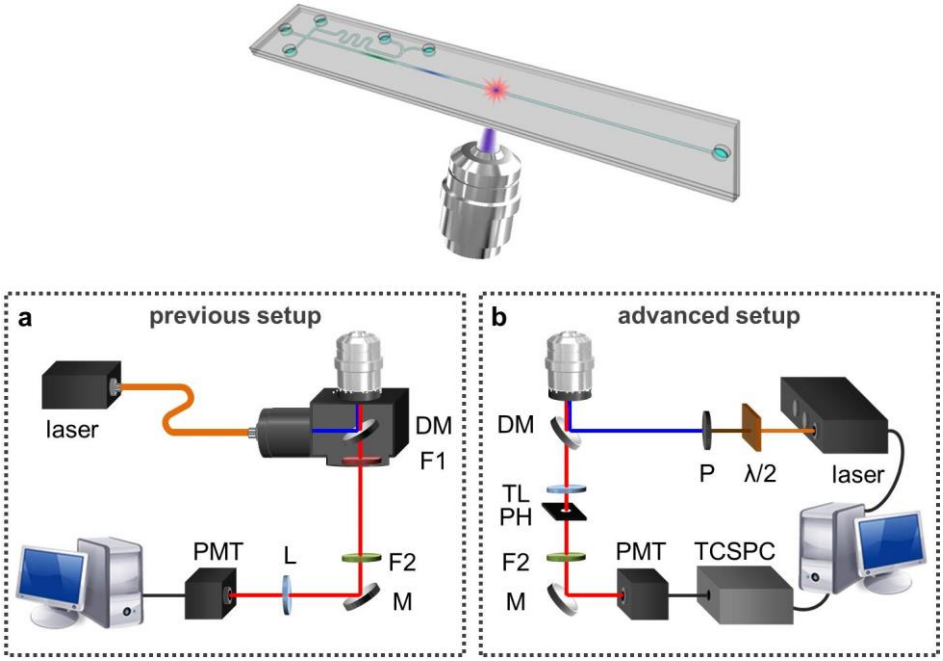

Figure S3. Schematic drawing of laser induced fluorescence detection in the deep-UV spectral region. a) Previous setup with fiber coupled laser. b) Significantly advanced detection setup with open beam laser and time-resolved detection option. Blue, excitation light (266 nm); red, emission light; DM, dichroic mirror (< 280 nm); F1, blocking filter (< 290 nm); F2, DUG11x emission filter (bandpass 280 nm to 370 nm); M, fused silica mirror; L, collector lens; TL, tube lens; PH, pinhole; P, polarizer; λ/d, half wave plate.

For advanced time-resolved label-free fluorescence detection with excitation in the deep UV spectral region, a setup based on the MicroTime 200 platform (PicoQuant, Berlin, Germany) and an Olympus IX71 microscope was used. As excitation source a picosecond pulsed Nd:YVO_4 (neodymium-doped yttrium orthovanadate) laser (Cougar, Time-Bandwidth Products, Zürich, Switzerland) with a repetition rate of 20 MHz adjusted at a laser power of 15 mW and operating at a wavelength of 266 nm was implemented.[4,5] The laser light was reflected by a dichroic mirror (< 280 nm) and focused onto the microfluidic channel by a 40x, 0.8 NA fused silica objective lens (Partec,

(4) Ohla, S.; Beyreiss, R.; Fritzsche, S.; Gläser, P.; Nagl, S.; Stockhausen, K.; Schneider, C.; Belder, D. *Chem. Eur. J.* **2012**, *18*, 1240-1246.
(5) Beyreiss, R.; Ohla, S.; Nagl, S.; Belder, D. *Electrophoresis* **2011**, *32*, 3108-3114.

Münster, Germany). Fluorescence light was collected by the same objective. After passing the dichroic mirror, emission light was sent to a photon counting photomultiplier tube (PMA 165-N-M, PicoQuant) synchronized to the laser frequency using a time-correlated single photon counting module. Additionally, a Schott DUG11x filter (bandpass 280 to 370 nm) was used for emission filtering (ITOS, Mainz, Germany). For electropherogram construction, binning time was set to 50 ms. Moreover, the integration frame was delayed by 5.9 ns with respect to the laser pulse to discriminate short-live background fluorescence and thereby improve the signal-to-noise ratio. The signal integrations were performed with the chromatography software Clarity (version 4.0, DataApex, Prague, Czech Republic). In case of not completely separated enantiomer's signals, peak areas were determined after manual integration using Clarity and the perpendicular drop method.

5. Limits of detection and quantitation

For the limit of detection (LOD) determinations, combined samples of substrate **1** and product **2** with concentrations of 4 µg/mL to 20 µg/mL were filled in SI for MCE-LIF. The LODs were calculated based on a five-point (n = 3) calibration curve (Figure S4a and b) and a signal-to-noise ratio (S/N) of 3 (Table S1).

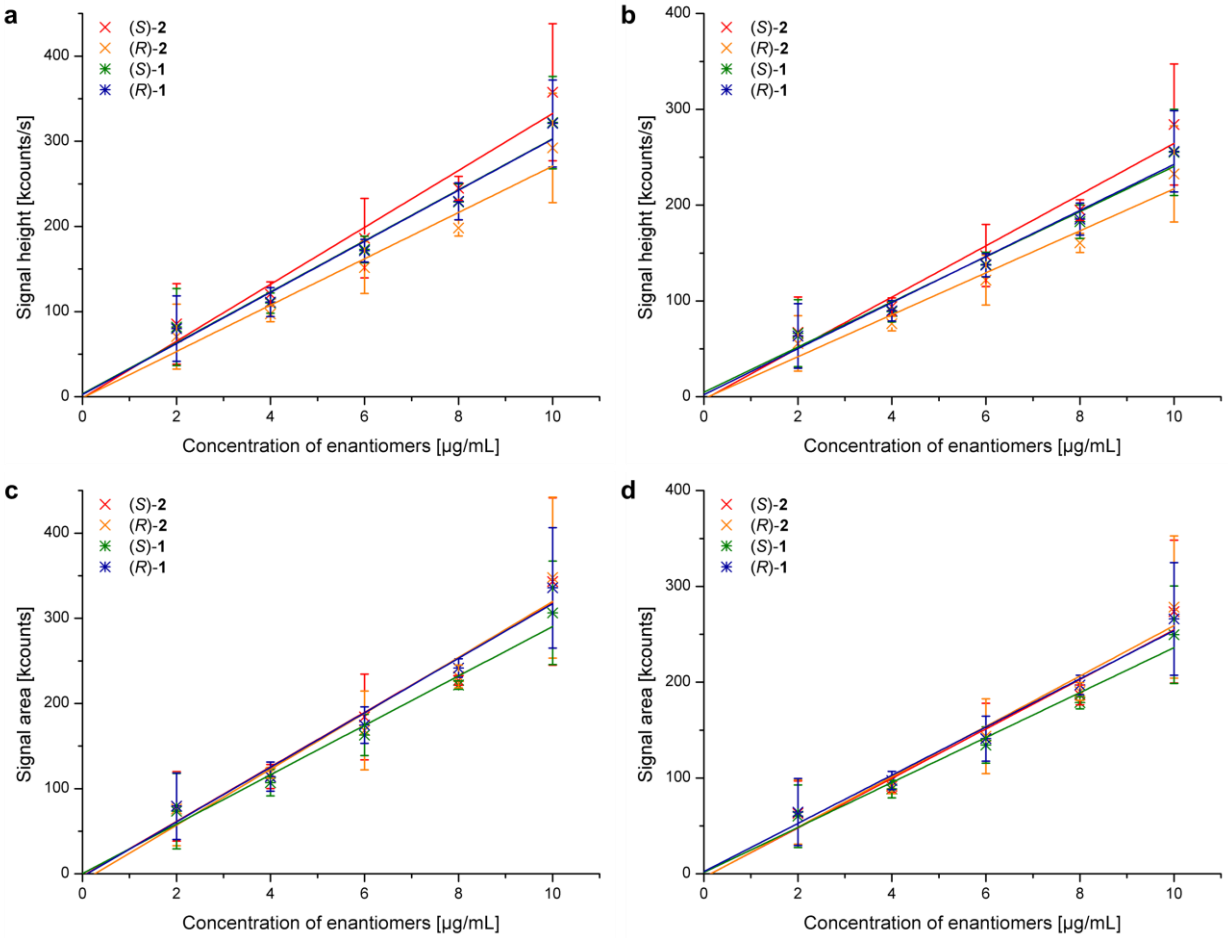

Figure S4. Five-point calibration curves (n = 3) with signal heights (a and b) and with signal areas (c and d) of enantiomers at different concentrations from 4 µg/mL to 20 µg/mL racemic substances **1** and **2**. a) and c) Without discrimination of background fluorescence via time frame. b) and d) With integration frame of 5.9 ns with respect to the laser pulse in order to discriminate short-live background fluorescence.

Table S1. Analytical values of substrate enantiomers **1** (molar mass: 150.174 g/mol[6]) and product enantiomers **2** (molar mass: 168.189 g/mol[6]) for measurements with previous MCE-LIF[2], and advanced MCE-LIF setup (with and without discrimination of short-live fluorescence < 5.9 ns). R^2, correlation coefficient of calibration curves (Figure S4); LOD, limit of detection; LOQ; limit of quantitation.

			Advanced setup	
		Previous setup[2]	**without fluorescence discrimination**	**with fluorescence discrimination**
Migration time / *s*	(*S*)-**2**		79 s ± 3 s	
	(*R*)-**2**		81 s ± 3 s	
	(*S*)-**1**		86 s ± 3 s	
	(*R*)-**1**		87 s ± 3 s	
***R*²**	(*S*)-**2**	0.997	0.949	0.950
	(*R*)-**2**	0.996	0.944	0.951
	(*S*)-**1**	0.999	0.958	0.959
	(*R*)-**1**	0.999	0.961	0.966
LOD / *µM*	(*S*)-**2**	23 ± 3	1.6 ± 0.2	0.9 ± 0.2
	(*R*)-**2**	26 ± 3	2.0 ± 0.3	1.1 ± 0.2
	(*S*)-**1**	26 ± 3	2.0 ± 0.3	1.1 ± 0.2
	(*R*)-**1**	26 ± 3	2.0 ± 0.3	1.1 ± 0.2
LOQ / *µM*	(*S*)-**2**	68 ± 9	4.9 ± 0.7	2.7 ± 0.5
	(*R*)-**2**	77 ± 10	6.0 ± 0.9	3.3 ± 0.6
	(*S*)-**1**	78 ± 10	6.1 ± 0.9	3.3 ± 0.6
	(*R*)-**1**	79 ± 10	6.1 ± 0.8	3.3 ± 0.6

(6) D. R. Lide, *CRC handbook of chemistry and physics*, 84th Ed., CRC Press, Boca Raton, op. **2003**, 3-462-3-466.

Measurements close to the calculated LOD were taken with a concentration of 0.6 µg/mL racemic **1** and **2** for MCE with advanced LIF detection (Figure S5).

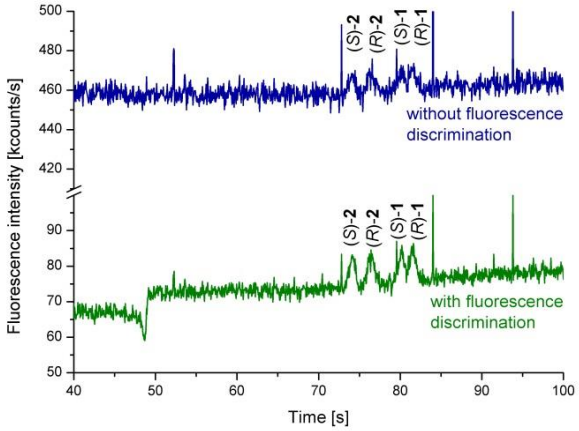

Figure S5. MCE-LIF measurement close to the calculated LOD with a concentration of 0.6 µg/mL racemic 1 and 2 with and without discrimination of short-live background fluorescence < 5.9 ns.

6. On-chip syntheses

Controls

To check whether the monitored reaction is catalyzed by whole cells and not by free enzymes in solution, control samples were formed based on cell suspensions of *E. coli* BL21-Gold(DE3) expressing ANEH-WT. The control samples included supernatants derived from centrifugation of cell suspension as well as filtrates of cell suspension. Then on-chip "reactions" in the sample inlet cavity with a volume of 7 µL, were performed with initial 20 µg/mL substrate **1** and initial $5.0 \cdot 10^5$ cells/µL (mixture 1:1 (v/v)) (Figure S6).

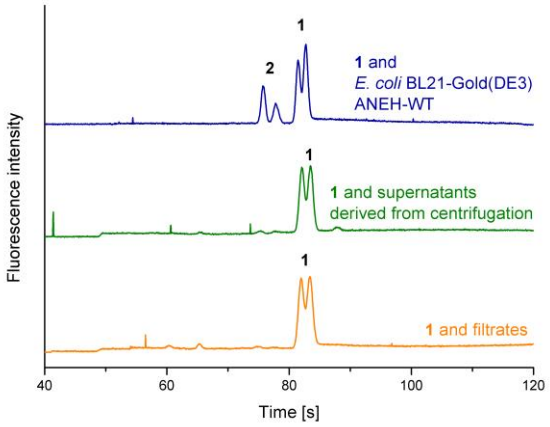

Figure S6. Electropherograms after on-chip reaction of substrate **1** hydrolysis catalyzed by *E. coli* BL21-Gold(DE3) expressing ANEH-WT to form product **2** (blue) as well as substrate **1** with control samples included supernatants derived from centrifugation (green) or filtrates (orange) of cell suspension using 0.2 µm pore size nylon syringe filters.

To prove that the monitored reaction is catalyzed by ANEH and not by any other present enzyme of *E. coli*, non-induced cells (without ANEH expression) as well as negative controls (substrate in PBS) were also applied for the on-chip reaction (Figure S7).

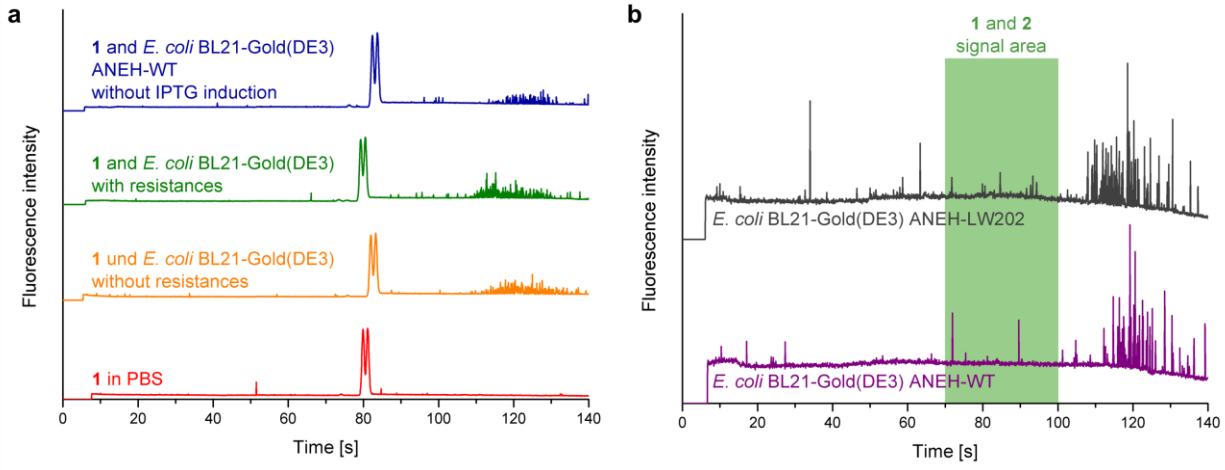

Figure S7. Electropherograms of a) substrate **1** in PBS (red), *E. coli* BL21-Gold(DE3) without (yellow) and with (green) antibiotics, without IPTG induction (blue) in PBS and b) *E. coli* BL21-Gold(DE3) expressing ANEH-WT (purple) or ANEH-LW202 (grey) in PBS.

Different reaction volumes and reduced cell number

Experiments for the comparison of reactions in different reaction volumes were performed with substrate **1** and *E. coli* BL21-Gold(DE3) cells suspension expressing ANEH-WT. Reactions in the sample inlet cavity with a volume of 7 µL, in the decreased reaction volume of 29 nL ± 2 nL (integrated microreactor) and in the further reduced reaction volume of 6.2 nL ± 0.4 nL (injection channel) were performed analogous as described above with initial 20 µg/mL substrate **1** and initial $5.0 \cdot 10^5$ cells/µL (mixture 1:1 (v/v)). The obtained signal-to-noise ratios (S/N) for the smallest signal ((*R*)-**2**) are quite similar for all reaction volumes and indicated that it could be possible to further reduce the cell number (Table S2).

Table S2. Signal-to-noise ratios (S/Ns) of (*S*)-product **2** and (*R*)-product **2** signals after successfully performed (S)-enantioselective whole-cell-based biocatalysis in different reaction volumes with initial $5.0 \cdot 10^5$ cells/µL *E. coli* BL21-Gold(DE3) cells expressing ANEH-WT and initial 20 µg/mL substrate **1** (mixture 1:1 (v/v)). Ø, average; SD, standard deviation.

Reaction volume	**Cell amount**	**S/N**	
		(*S*)-**2**	(*R*)-**2**
7 µL	$1.7 \cdot 10^6 \pm 0.3 \cdot 10^6$	103	55
29 nL	7000 ± 1500	114	64
6.2 nL	1500 ± 300	67	47
Ø		95	55
SD		25	9

More determinations were performed in the reduced reaction injection volume with 20 µg/mL **1** and decreased cell concentration of the initial $1.3 \cdot 10^5$ cells/µL (mixture 1:1 (v/v)), which leaded to small S/N values close to the LOQ (S/N of 9) (Table S3).

Table S3. Signal-to-noise ratios (S/Ns) of (*S*)-product **2** and (*R*)-product **2** signals after successfully performed (S)-enantioselective whole-cell catalyses in the smallest reaction volume of 6.2 nL with 390 ± 80 *E. coli* BL21-Gold(DE3) ANEH-WT and initial 20 µg/mL substrate **1** (n = 3). Ø, average; SD, standard deviation.

Measurement	**S/N**	
	(*S*)-**2**	(*R*)-**2**
1	26	15
2	31	19
3	25	10
Ø	27	15
SD	3	5

Calculation of minimal activity

The theoretical calculations for the minimal activity of ANEH-WT in *E. coli* BL21-Gold(DE3) were performed using the minimal number of cells (390 cells in a reaction volume of 6.2 nL). The amount of product **2** in the reaction volume was calculated based on the signal area and the calibration curve (Figure S4d). Table S4 summarizes the minimal enzyme activity per cell. Using 390 cells, the introduced setup can detect 1.22 pmol product per min. However, because ANEH is expressed poorly in *E. coli* and it is difficult to quantify it (Figure S8), we were unable to determine specific enzyme activity.

Table S4. Concentration and mass in reaction volume (6.2 nL) of product **2** based on the signal area and the calibration curves after on-chip enantioselective catalytic hydrolysis of substrate **1** by minimal number of *E. coli* BL21-Gold(DE3) cells expressing ANEH-WT (390 cells) with a reaction time of 180 s.

Calculated concentrations / *µg/mL*		**Mass in reaction volume /** *ng*		**Total molar quantity /** *pmol*	**Enzyme units /** *fmol/cell/min*
(*S*)-**2**	(*R*)-**2**	(*S*)-**2**	(*R*)-**2**		
69	31	0.43	0.19		
		Σ 0.62		3.7	3.1

Expression levels of ANEH-WT and ANEH-LW202

To study the expression levels 10 µL of each culture – overexpressing ANEH-WT or ANEH-LW202 with normalized OD_{600}-values – were used to perform SDS-PAGE. We used commercially available 4 to 15% Mini-Protean TGX precast gels and the proteins were stained with Coomassie G-250 dye available in a commercial solution. The theoretical size of ANEH is ca. 45 kDa (GI: 7245813). As shown in Figure S8, however, we could not see the expected bands. To better judge the enzyme amount, we included the commonly used BSA standards with known concentrations. As it can be seen in the gel, the lowest amount of protein that can be detected in the assayed conditions is 500 ng BSA. Thus, it can be said that the ANEH enzymes are expressed below 50 ng/µL.

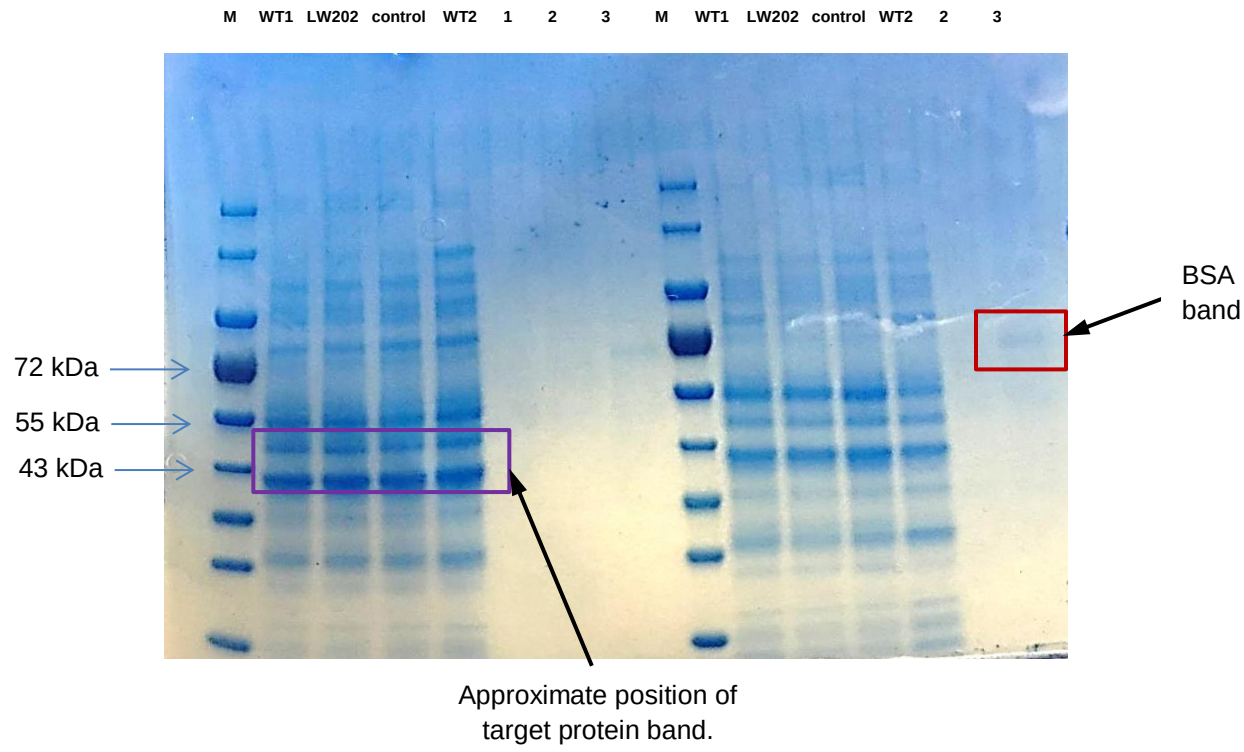

Figure S8. SDS-PAGE of *E. coli* BL21-Gold(DE3) overexpressing ANEH-WT or ANEH-LW202. M, marker; WT1, *E. coli* expressing ANEH-WT with two plasmids system; LW202, *E. coli* expressing ANEH-LW202 with two plasmids system; control, *E. coli* containing the two empty plasmids; WT2, *E. coli* expressing ANEH-WT with single plasmid (no pREP4); Lane 1, BSA standard with concentration of 10 ng/µL; Lane 2, BSA standard with concentration of 25 ng/µL; Lane 3, BSA standard with concentration of 50 ng/µL.

Lysates and whole cells

On-chip reactions in PBS buffer were performed by mixing substrate **1** 40 µg/mL and cell suspension in the sample inlet cavity or inside the microchannels 1:1 (v:v). After a reaction time of 180 s a small aliquot of the reaction mixture was transferred to the separation channel by a voltage-controlled pinched injection scheme and analyzed by chiral chip electrophoresis. This procedure was applied for experiments with *E. coli* BL21-Gold(DE3) expressing either ANEH-WT or ANEH-LW202 (Figure S9).

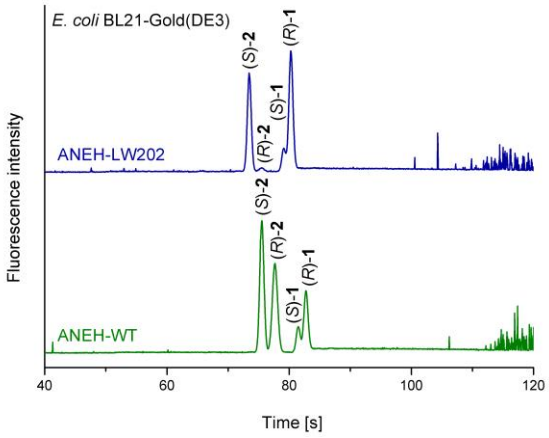

Figure S9. Electropherograms after on-chip enantioselective catalytic hydrolysis of substrate **1** to form product **2** by *E. coli* BL21-Gold(DE3) cells expressing ANEH-WT or mutant LW202.

For comparison reactions with cell lysates were performed (Figure S10). Therefore, 5 µL of lysozyme in water (100 mg/mL) were added to 495 µL of cell suspensions of *E. coli* BL21-Gold(DE3) ANEH-WT or ANEH-LW202 in PBS buffer in Eppendorf vials. This was shaked at 37 °C for 2 h. Then on-chip reactions with the obtained lysates were performed analogous to the method with whole cells described above.

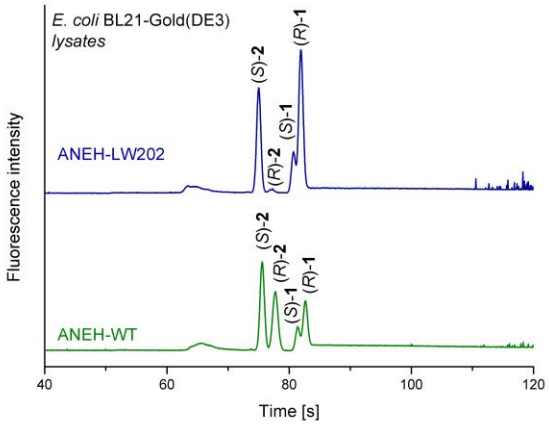

Figure S10. Electropherograms after on-chip enantioselective catalytic hydrolysis of substrate **1** to form product **2** by *E. coli* BL21-Gold(DE3) ANEH-WT or ANEH-LW202 lysates.

Different substrates

Additionally, experiments with different epoxide substrates were performed analogous to the procedure described above using *E. coli* BL21-Gold(DE3) ANEH-WT or ANEH-LW202 (Figure S11). The initial concentrations of substrate **3a**, **3b**, and **3d** were 40 µg/mL. For substrate **3c** a 400 µg/mL solution was utilized. All of these substrates were diluted in PBS buffer with 2% acetonitrile.

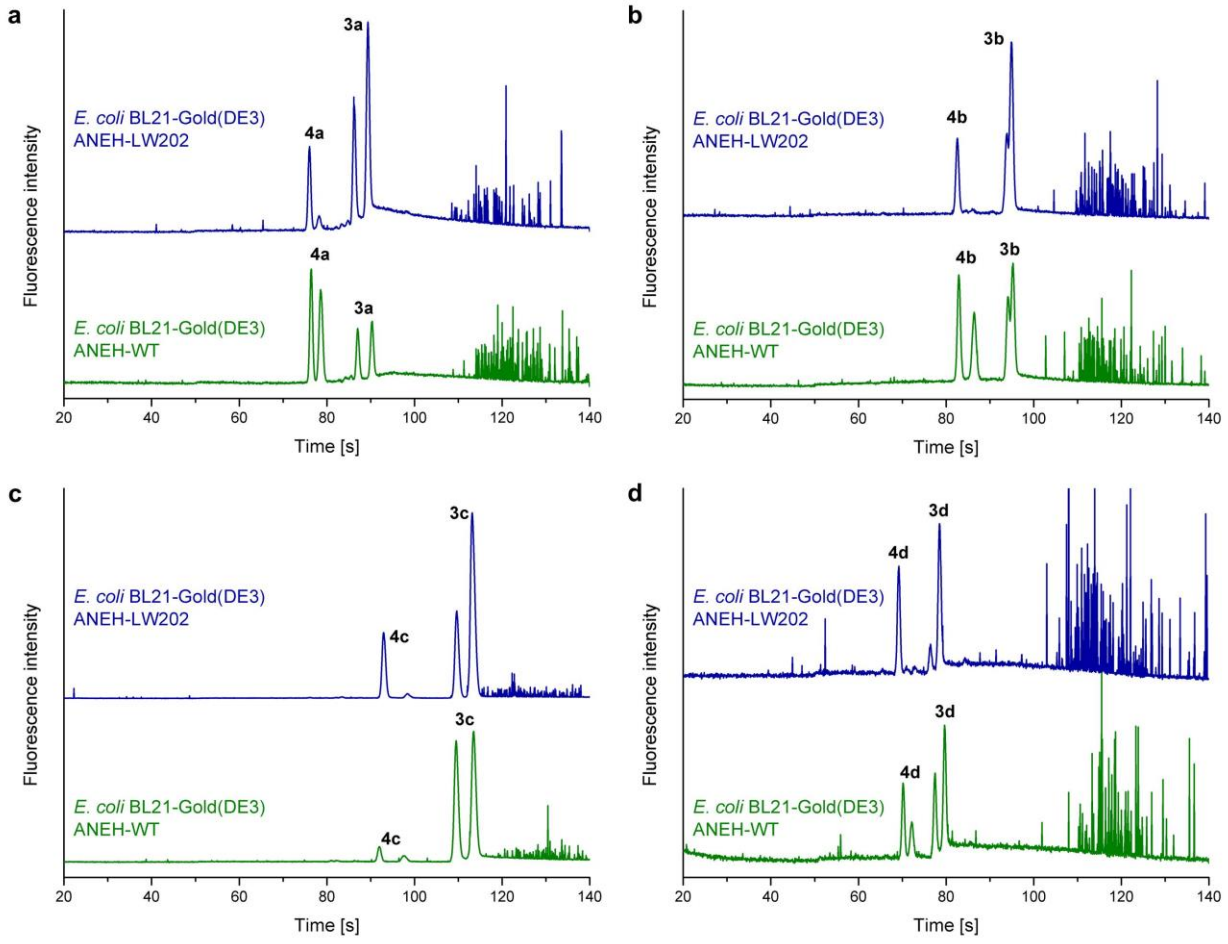

Figure S11. Electropherograms after on-chip enantioselective catalytic hydrolysis of a) **3a**, b) **3b**, c) **3c** and d) **3d** to by *E. coli* BL21-Gold(DE3) ANEH-WT or ANEH-LW202.